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# Diagnosis of the Hemolytic State Using Serum Levels of Erythrocyte Adenylate Kinase

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**Red cell hemolysis is classically diagnosed by a combination of nonspecific laboratory tests, including serum bilirubin, LDH, and the reticulocyte count. None of these tests alone or in combination has the specificity to reliably ascertain the presence of hemolysis. We have previously demonstrated that erythrocyte adenylate kinase (EAK) is a red cell specific enzyme released from damaged red cells. Its activity can be measured in serum by rapid electrophoresis or immunological methods and correlates linearly with the degree of hemolysis in vitro. We now report on a clinical study comparing EAK levels in patients with and without hemolysis. The clinical diagnosis of hemolysis was established in hospitalized patients with anemia by the combined elevation of the bilirubin, LDH, and reticulocyte count in the absence of liver disease and demonstrable blood loss. The normal range of serum EAK was determined in 30 healthy nonanemic voluntary blood donors and was 0–3.5 Units (mean = 0.5). In 25 patients with hemolytic anemia due to sickle cell disease, hemolytic transfusion reactions, or TTP, the mean EAK level was 62.4 with a range 0–298 Units ($P < 0.001$ compared to normals). Levels of EAK exceeded the normal range in 24 of 25 patients (96%). In a control group of 44 hospitalized patients with liver disease or myocardial infarction and no clinical evidence of hemolysis, the mean EAK level was 0.12 with a range of 0–3.2 ($P = 0.1$, NS compared to normals and $P < 0.001$ compared to patients with hemolysis). None of the control patients had EAK levels that exceeded the normal range. The diagnostic sensitivity of the EAK assay for hemolysis, as calculated according to Baye's algorithm, was 96%, with a specificity and accuracy of 97%. Measurement of serum EAK represents a highly sensitive and specific test for the diagnosis of hemolytic anemia. Am. J. Hematol. 64:180–183, 2000.**


Key words: hemolysis; anemia; adenylate kinase

## INTRODUCTION

Hemolysis as the cause of anemia is readily identified by the combined findings of increased red cell destruction and production. Markers of accelerated erythrocyte destruction include serum levels of unconjugated bilirubin, lactate dehydrogenase (LDH), and haptoglobin. The bilirubin level is both insensitive, being normal in almost half of patients with immune hemolytic anemia [1], and nonspecific, being elevated in patients with liver dysfunction [2]. LDH levels are most reliably elevated in patients with intravascular hemolysis [3] or ineffective erythropoiesis [4], but they are inconsistently variable in conditions of extravascular hemolysis. The serum haptoglobin level is the most sensitive test for hemolysis but remains nonspecific because this protein is an acute phase reactant that can rise to normal levels even in the presence of hemolysis when inflammatory conditions are present [5]. Other tests such as the plasma hemoglobin and urine hemosiderin have even less clinical utility, while the $^{51}$chromium red cell survival test, a clear gold standard for accelerated red cell destruction, is too cumbersome for routine rapid use. The reticulocyte count, which is an indicator of erythropoietic activity, is often elevated in hemolytic conditions, but it is frequently depressed in patients with abnormal marrow production, making it too insensitive an indicator of hemolysis.

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We have developed an assay for the measurement of erythrocyte adenylate kinase, an enzyme specifically found in the red cell cytosol. This enzyme is present at very low levels in normal human serum and is released into the serum in direct proportion to the number of red cells that are destroyed during hemolytic processes. We describe this assay and a clinical study demonstrating its specific utility in the diagnosis of hemolytic diseases.

## MATERIALS AND METHODS

### Erythrocyte AK Assay

The assay for measuring erythrocyte AK was performed using a modification of our previously described method [6]. The Cobas-Fara analyzer (Roche Diagnostics Systems, Somerville, NJ) was used to assay total AK activity in serum, using a 10-fold dilution of the AK visualizing reagent [6]. The AK activity was visualized as fluorescence of the NADH product formed by isozyme activity as follows:

$$2ADP \xrightarrow{\text{Adenylate kinase}} ATP + AMP,$$

$$ATP + \text{D-Glucose} \xrightarrow{\text{Hexokinase}} \text{D-Glucose-6-phosphate},$$

$$\text{D-Glu-6-PO}_4 + NAD \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-Phosphogluconate} + NADH.$$

The Helena REP CK electrophoresis instrument (Beaumont, TX) and CK isoenzymes fractionating agarose gels were used to fractionate the erythrocyte AK isoenzyme. The samples for analyses were applied to the gel, allowed to penetrate for 1 min, after which electrophoresis was performed at 1,200 volts for 2.4 min at 10°C. After electrophoresis the gel was incubated with the visualization reagent for 5 min at 45°C and dried at 54°C for 5 min. The percent erythrocyte AK activity was computed by scanning the 360-nm fluorescent spot of the NADH produced as a result of isoenzyme activity. The erythrocyte AK activity was quantified by multiplying the percent erythrocyte activity by the total AK activity of the sample.

### Patient Studies

The diagnosis of the hemolytic state was made by clinical criteria in the patients studied based on a definition of falling hemoglobin, increased reticulocyte count, and elevated conventional markers of red cell destruction. Hemoglobin and reticulocyte counts were measured on the STKS hematology analyzer equipped with flow cytometric reticulocyte module (Coulter, Miami, FL). Serum total bilirubin and lactate dehydrogenase levels were measured by conventional techniques on a Hitachi 914 chemistry analyzer (Boehringer). For a normal control group, blood was obtained from 30 healthy men and women who consented to have an additional blood sample drawn during a routine voluntary blood donation. A hemolysis group consisting of 25 patients with clinical hemolysis was identified and studied. The group consisted of 19 patients with chronic hemolysis, all having sickle cell anemia, and 6 patients with acute hemolysis, 2 patients with TTP, 2 with delayed hemolytic transfusion reactions, and 2 with autoimmune hemolytic anemia. The sickle cell patients had hemolysis as a definition of their disease. The six other patients in the hemolysis group were clinically classified as having accelerated red cell destruction on the basis of a rapidly falling hemoglobin in the absence of any discernible blood loss. In addition, two additional control groups, one consisting of 23 patients with acute myocardial infarction serving as an acutely ill hospitalized patient control group, and one 21-patient group with an assortment of conditions causing hepatic dysfunction serving as a control group with abnormal liver function. Both of these latter groups contained patients with stable anemia and no clinical evidence of accelerated red cell destruction. These two groups were thus classified as sick patients without clinical hemolysis.

## RESULTS

The group of patients with hemolysis had a mean EAK level of 62.4 (32.3–92.5, 95% CI) compared to a mean of 0.5 (0–1.03, 95% CI) in the Normal group ($P$ = 0.000005). Within the hemolysis group there was no significant difference between the EAK levels of patients with acute hemolysis (mean of 73.1 ± 77.2) and those with chronic hemolysis (mean of 58.7 ± 78.4, $P$ = 0.35).

The Acute M.I. group had a mean EAK level of 0.06 (0–0.18, 95% CI; $P$ = 0.003) and the Medically Sick group had a level of 0.2 (0–0.49, 95% CI; $P$ = 0.006). There was no overlap in the EAK levels between the hemolysis group and any of the others studied (Fig. 1). The sensitivity, specificity, and likelihood ratios of the EAK assay for the diagnosis of hemolysis is compared to those of LDH, T. bilirubin, and reticulocytes in Table I. Using the upper limit of the 95% confidence interval (CI) for each marker, the EAK assay had the best combined sensitivity (96%) and specificity (97%). Receiver operating characteristic (ROC) analysis of the various markers demonstrated that EAK had the best diagnostic utility (Fig. 2). Table II presents the mean values for the various markers and their 95% CI values. Unlike the EAK assay, which shows no overlap between the hemolysis group and the two hospitalized control groups, the LDH and T. bilirubin assays have a range of values that overlaps among diagnostic groups, thus limiting their clinical utility.

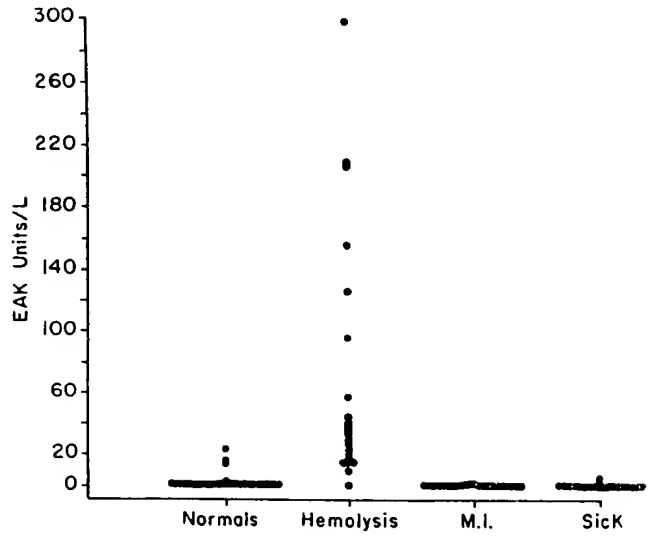


**Fig. 1. Dot-plot analysis of EAK levels in normals and patients with different disease states. Each dot represents a single patient.**

**TABLE I. Clinical Utility of Various Markers of Hemolysis**

| | Sensitivity | Specificity | Likelihood Ratio | −Likelihood ratio |
|---|---|---|---|---|
| Erythrocyte AK | 96 | 97 | 35.5 | 0.04 |
| LDH | 100 | 59 | 2.42 | 0 |
| T. bilirubin | 39 | 50 | 0.78 | 1.22 |
| % Reticulocytes | 100 | 85 | 6.8 | 0 |

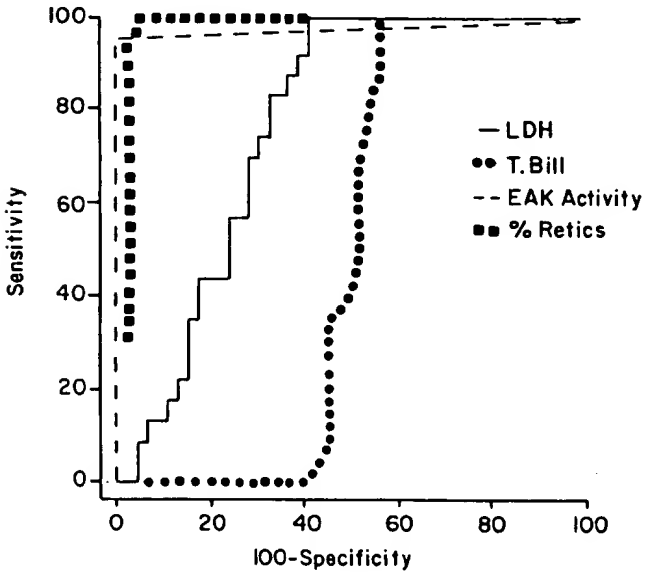


**Fig. 2. Receiver operator characteristics (ROC) analysis of diagnostic sensitivity versus specificity of different tests for the diagnosis of hemolysis.**

**TABLE II. Comparison of Blood Markers for Detecting Hemolysis**

| | Hemolysis | Acute M.I. | Medically Ill |
|---|---|---|---|
| Erythrocyte AK | 62.4 (32.3–92.5) | 0.06 (0–0.18) | 0.2 (0–0.49) |
| LDH | 770 (51–1489) | 524 (293–755) | 398 (229–566) |
| T. bilirubin | 2.1 (1.63–2.57) | 1.0 (0.7–1.3) | 6.0 (2.9–9.1) |
| % Reticulocytes | 12.2 (5.1–19.3) | 1.1 (0.97–1.23) | 1.2 (0.99–1.41) |

## DISCUSSION

The clinical diagnosis of hemolytic anemia is often obvious and should pose little diagnostic challenge in the context of known hematologic disease. Often, though, patients with complex medical problems display the signs or symptoms of anemia, requiring a comprehensive work-up. While the finding of an elevated reticulocyte count is a good indicator of an active bone marrow response to blood loss or hemolysis, this indicator is often blunted in the face of chronic disease, renal failure, chemotherapy, radiotherapy, or bone marrow dysfunction. The absence of reticulocytosis cannot reliably exclude hemolysis. In the present study, an elevated reticulocyte count had a sensitivity of 100% and a specificity of 85%. However, none of the subjects studied in this cohort of patients had blood loss that causes reticulocytosis or bone marrow suppression that lowers the reticulocyte count. Thus, in actual clinical practice where patients with AIDS and cancer abound, the reticulocyte count would be expected to have far less sensitivity. Similarly, nonspecific serum markers of hemolysis such as LDH and bilirubin are often elevated due to liver abnormalities in the very population where the question of hemolysis is raised, making their clinical utility limited. Our study confirms the low specificity of LDH (59%) and bilirubin (50%). It is therefore not surprising that a red cell specific enzyme that is released only when red cells are destroyed would be expected to provide greater clinical diagnostic efficacy.

Adenylate kinase is an enzyme involved in phosphoryl transfer between ATP and AMP in $K^+$ sensitive ATP channels [7]. It exists in at least five isoenzyme forms and is found in blood erythrocytes, brain, skeletal, and cardiac muscle [8]. The current methodology for quantitating EAK clearly distinguishes between erythrocyte specific adenylate kinase and adenylate kinases originating from other tissues [9]. Our method used the Cobas Fara instrument for total AK, but it can be easily adapted for use on any programmable random access chemistry analyzer. In addition we utilized NAD in place of NADP for the assay because it gave equivalent results and was more cost-effective. The assay as currently performed, though, is somewhat cumbersome making full automation desirable for more widespread use. We are currently developing an immunoassay methodology for EAK that should simplify this goal.

We have demonstrated that EAK is a highly sensitive and specific marker for erythrocyte hemolysis. As such, its clinical utility in the diagnosis of anemia would be to differentiate between disorders of red cell destruction and those of inadequate production or blood loss. This would be especially critical when it points the way towards hemolytic conditions requiring emergent diagnosis and therapy such as autoimmune hemolytic anemia and thrombotic thrombocytopenic purpura. While the sensitivity of the test was no better than the reticulocyte count in this study, its specificity was superior. Furthermore, it is well known that the reticulocyte count has poor sensitivity for hemolysis in other patient populations whose erythropoietic response is known to be blunted. The use of the EAK assay early on in a diagnostic algorithm for anemia diagnosis should serve to reduce the total number of tests performed and speed the determination of the etiology of the anemia.

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